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Supplemental Assay Method for the Titration of Feline Rhinotracheitis Virus in Cell Culture

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* titration method for assaying modified-live feline rhinotracheitis virus (FRV) vaccines for viral content. The method uses plaque-forming units (PFU) in a cell culture system for titration of FRV.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Syringe, self-refilling, repetitive, 2-mL

2.1.2 Micropipettor, 200-µL, and tips (Rainin Pipetman®) or Handistep

2.1.3 Blender

2.1.4 Media bottle, borosilicate glass with screw-top lid, 1000-mL

2.1.5 Incubator, 36°± 2°C, high-humidity, 5% ± 1% CO_2 (Model 3158, Forma Scientific Inc.)

2.1.6 Water bath

2.1.7 Inverted light microscope (Model CK, Olympus America Inc.)

2.1.8 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.9 Pipette-aid

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 FRV Positive control, C-27 strain (available from the Center for Veterinary Biologics [CVB])

2.2.2 Crandall feline kidney (CRFK) cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR) by 113.52

2.2.3 Minimum essential medium (MEM) (National Veterinary Services Laboratories [NVSL] Media # 20030

1. 9.61 g MEM with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate ($NaHCO_3$)

3. Dissolve with 900 mL deionized water (DW).

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DW. Heat to 60°± 2°C until dissolved. Add to **Step 3** with constant mixing.

5. Q.S. to 1000 mL with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

6. Sterilize through a 0.22-µm filter.

7. Aseptically add 50 µg/mL gentamicin sulfate.

8. Store at 2°- 7°C.

2.2.4 Growth Medium

1. 900 mL of MEM

2. Aseptically add:

a. 100 mL gamma-irradiated fetal bovine serum (FBS)

b. 10 mL L-glutamine (200 mM)

3. Store at 2°- 7°C.

2.2.5 2X Medium

1. 19.22 g MEM with Earles salts without bicarbonate

2. 2.2 g $NaHCO_3$

3. Dissolve with 900 mL DW.

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DW. Heat to 60°± 2°C until dissolved. Add to **Step 3** with constant mixing.

5. Q.S. to 1000 mL with DW, and adjust pH to 6.8-6.9 with 2N HCl.

6. Sterilize through a 0.22-µm filter.

7. Store at 2°- 7°C.

8. Prior to preparing the Overlay Medium (**Section 3.3.3**), aseptically add 100 µg/mL gentamicin sulfate.

2.2.6 2% Tragacanth Gum (Trag)

1. 20 g Trag

2. 1000 mL DW

3. Add small amounts of Trag at a time and vigorously mix with a blender set on high.

4. Pour 500 mL into individual glass 1000-mL media bottles.

5. Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

6. Store at 2°- 7°C.

2.2.7 7.5% Sodium Bicarbonate

1. 7.5 g $NaHCO_3$

2. Q.S. to 100 mL with DW.

3. Sterilize by autoclaving at 15 psi, 121°± 2°C for 30 ± 10 minutes.

4. Store at 2°- 7°C.

2.2.8 70% Ethyl Alcohol (NVSL Media # 30184)

1. 73 mL ethyl alcohol (95% [195 proof])

2. 27 mL DW

3. Store at room temperature.

2.2.9 Crystal Violet Stain (NVSL Media # 30012)

1. 7.5 g crystal violet

2. 50 mL 70% Ethyl Alcohol

3. Dissolve crystal violet in alcohol; add remaining ingredients.

4. 250 mL formaldehyde

Note: According to 29 CFR 1910.1048, precautions for the use of formaldehyde are as follows: Toxic by inhalation and if swallowed. Irritating to the eyes, respiratory system and the skin. May cause sensitization by inhalation or by skin contact. Risk of serious damage to eyes. Potential cancer hazard; repeated or prolonged exposure increases the risk.

5. Q.S. to 1000 mL with DW.

6. Filter through a Whatman® #1 filter.

7. Store at room temperature.

2.2.10 Tissue culture plates, 4-well

2.2.11 Polystyrene tubes, 12 x 75-mm

2.2.12 Pipettes, 25-mL

2.2.13 Needles, 18-gauge x 1 1/2-inch

2.2.14 Syringes, (tuberculin) 1-mL

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantification of virus infectivity by plaque formation.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK tissue culture plates (Test Plates)

Cells are prepared from healthy, confluent CRFK cell cultures that are maintained by passing every 3 to 4 days. Two days prior to test initiation, add 8.0 ml/well of approximately $10^{5.0}$ to $10^{5.3}$ cells/mL suspended in Growth Medium into all wells of the 4-well tissue culture plate. Prepare one Test Plate for the FRV Positive Control and one for each Test Vaccine. Incubate at 36°± 2°C in a CO_2 incubator.

3.3.2 Preparation of FRV Positive Control

1. On the day of test initiation, rapidly thaw a vial of the current FRV Positive Control virus in the water bath.

2. Dispense 1.8 mL of MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB Reagent Data Sheet; appropriately label (e.g., 8 tubes, labeled 10^{-1} through 10^{-8}, respectively).

3. Transfer 200 µL of the FRV Positive Control virus to the tube labeled 10^{-1}; mix by vortexing.

4. Using a new pipette tip, transfer 200 µL from the 10^{-1}-labeled tube to the 10^{-2} tube; mix by vortexing.

5. Repeat **Step 4** for each of the subsequent dilutions, transferring 200 µL of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

3.3.3 Overlay Medium Preparation

On the day of test initiation, prepare approximately 35 mL per plate of Overlay Medium. Volumes listed are for 1 liter of Overlay Medium.

1. Aseptically add to 500 mL of 2X Medium:

a. 10% gamma-irradiated FBS

b. 10 mL of 7.5% Sodium Bicarbonate

c. 50 µg/mL gentamicin sulfate

d. 50 mL of 2% Trag

2. Mix and warm the Overlay Medium in a 36°± 2°C water bath for 60 ± 10 minutes prior to performing the procedure in **Section 4.5**.

3.4 Preparation of the Test Vaccine

3.4.1 The initial test will be with a single vial (a single sample from 1 vial). On the day of test initiation, using a 1.0-mL syringe and an 18-gauge x 1 1/2-inch needle, rehydrate a vial of the Test Vaccine with the provided diluent by transferring 1.0 mL for a 1-mL-dose vaccine, 0.5 mL for a 1/2-mL-dose vaccine, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 Dispense 1.8 mL MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6} respectively, using a 2-mL self-refilling, repetitive syringe.

3.4.3 Transfer 200 µL of the Test Vaccine to the tube labeled 10^{-1}; mix by vortexing.

3.4.4 Using a new pipette tip, transfer 200 µL from the tube labeled 10^{-1} to the 10^{-2} tube; mix by vortexing.

3.4.5 Repeat **Section 3.4.4** for each of the subsequent dilutions, transferring 200 µL of the previous dilution to the next dilution tube until the tenfold dilution series is completed.

4. Performance of the Test

4.1 On the day of test initiation, label the Test Plates and remove the Growth Medium.

Note: Presence of confluent cells is necessary in order to distinguish plaques due to infection by FRV. If cells are not confluent at two days incubation, start of the test may be delayed an additional day in order to obtain confluency.

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4.2 Inoculate 1 well/dilution with 200 µL from dilutions 10^{-6} through 10^{-3} of the Test Vaccine. Inoculate 1 well/dilution from dilutions 10^{-8} through 10^{-6} of the FRV Positive Control. Change tips between each unique sample (i.e., each Test Vaccine), but tip changes are not necessary between each dilution in a series when pipetting from the most dilute to the most concentrated (e.g., 10^{-8} through 10^{-6}). Gently rotate the plates to evenly disperse the inoculum.

4.3 One uninoculated well on a Test Plate serves as a negative cell control.

4.4 Incubate the inoculated Test Plates at 36°± 2°C for 75 ± 15 minutes in a CO_2 incubator. To evenly disperse the inoculum, gently rotate the plates at intervals of 25 ± 5 minutes.

4.5 Add 8 mL/well of the Overlay Medium to the plates with a 25-mL pipette. Discard any unused, warmed Overlay Medium.

4.6 Incubate the Test Plates undisturbed at 36°± 2°C in a CO_2 incubator for 4 days ± 1 day.

4.7 After incubation, without removing the Overlay Medium, pipette 5 mL of the Crystal Violet Stain into each well of the Test Plates with a 25-mL pipette; mix by gentle rotation. **Note the precaution statement in Section 2.2.9(4).**

4.8 Allow Test Plates to incubate at room temperature for 25 ± 5 minutes.

4.9 Wash the Overlay Medium and the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container overflowing with running water from the cold water tap until the water is clear. Allow to air dry.

4.10 Count the number of FRV plaques for each well and record observations. If FRV and feline calicivirus (FCV) plaques are counted together in a combination vaccine, the FRV plaques will contrast markedly from FCV. The FCV plaques are large, clear circular areas (averaging 3 to 4 mm in diameter) with fuzzy edges. The FRV plaques are visible as small, clear plaques approximately 1 mm diameter with distinct edges. If the plaques are not distinguishable by size, use an inverted light microscope with 100X magnification to differentiate the plaque edges.

4.11 Record results as the number of FRV plaques for each dilution of a Test Vaccine and the FRV Positive Control.

4.12 Calculate the FRV titers of the Test Vaccine and the FRV Positive Control from the dilutions with plaque counts containing 10-100 plaques. The titer may be expressed as PFU per dose of vaccine.

Example:

During the titration of a Test FRV vaccine, the plaque count obtained at dilution 10^{-3} was 65. Calculation of the titer of this vaccine is done according to the following formula:

Test Vaccine Titer = X + p, where

X = Log_{10} of plaque count
p = Log_{10} of dilution counted

In the above example:

Log_{10} of plaque count (65) = 1.8
Log_{10} of dilution counted (10^{-3}) = 3.0

FRV Test Vaccine Titer = 1.8 + 3.0 = 4.8

Adjust the titer to the recommended Test Vaccine dose as follows:

A. Divide the **Test Vaccine** Dose by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this test FRV vaccine is 1 mL)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this test FRV vaccine, the inoculation dose is 0.2 mL)

$$\frac{\text{1 mL dose}}{\text{0.2 mL inoculum}} = 5$$

B. Calculate log_{10} of value in **A** and add it to the **Test Vaccine Titer** as illustrated below:

Log of 5 = 0.7

FRV Test Vaccine Titer = 4.8 + 0.7 = 5.5

Therefore, the titer of the **FRV Test Vaccine** is $10^{5.5}$ PFU_{50}/mL.

4.13 Conversion from PFU_{50}/mL to $TCID_{50}$/mL.

One plaque represents a **single infective unit** (IU), whereas one, 50% tissue culture infective dose ($TCID_{50}$) is statistically equivalent to a theoretical 0.69 IU. A $TCID_{50}$ endpoint per dose will be 1.44 times those expressed as PFU per dose. Therefore, to

express a PFU titer as a $TCID_{50}$ titer, multiply the plaque count by 1.44. In the example above, multiply 65 times 1.44 resulting in 93.6. Convert 93.6 to $\log_{10}$ resulting in 1.97. Add the log for the dilution counted (3) and the conversion factor for dose (0.7) which results in a $\log_{10}$ $TCID_{50}$/mL of 5.67. In practical use, 0.16 (the log of 1.44) may be added to the $\log_{10}$ value of the PFU titer per mL to convert to $TCID_{50}$/mL. In the above example, the titer of the Test Vaccine is $10^{5.66}$ $TCID_{50}$/mL.

5. Interpretation of the Test Results

5.1 Validity requirements

5.1.1 The calculated titer of the FRV Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell control cannot exhibit any plaques, cytopathic effect (CPE), or cloudy media that would indicate any contamination.

5.1.3 For endpoint determination, only those tenfold dilutions of the Test Vaccine and the FRV Positive Control inducing 10 to 100 plaques are included in the plaque counts.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY**.

5.4 In a valid test, if the titer of the Test Vaccine is less than the titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine may be retested in accordance with the Code of Federal Regulations, Title 9, Part 113.8(b).

6. Report of Test Results

Results are reported as PFU_{50} or $TCID_{50}$ per dose of Test Vaccine.

7. References

7.1 Code of Federal Regulations, Title 9, Parts 113.8(b) and 113.315, U.S. Government Printing Office, Washington, DC.

7.2 Code of Federal Regulations, Title 29, Part 1910.1048, U.S. Government Printing Office, Washington, DC.

7.3 Cottral, GE, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates. Ithaca, NY, 1978, pg. 731.

7.4 Davis, Dulbecco, Eisen, and Ginsberg, ed., *Microbiology including Immunology and Molecular Genetics*, 3rd ed. Harper and Row, Hagertown, MD, 1980, pg 880.

8. Summary of Revisions

Version .03

- The Contact has been changed from Victor Becerra to Alethea Fry.
- **2.2.9:** A precautionary statement for use of formaldehyde has been added.
- **4.13:** Conversion for PFU_{50}/mL to $TCID_{50}$/mL has bee clarified.
- Use of reference control terminology has been changed to positive control throughout the document.

Version .02

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **1.2** "Key Words" has been deleted.
- **2.2.3.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.
- **2.2.5.8** 50 µg/ml gentamicin sulfate has been changed to 100 µg and usage of penicillin and streptomycin has been deleted.
- **4.6** Ninety-six hours has been changed to 4 days ± 1 day.

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- **4.12** The determination of virus titer for each Test Vaccine has been rewritten to correct errors in calculations using the example given.

- **5.1.3** The endpoint determination of plaques has been clarified.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.

- "Test Serial" has been changed to "Test Vaccine" throughout the document.

- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.

- The footnotes have been deleted with any pertinent references now noted next to the individual items.